OBSERVATIONS ON THE CENTRIFUGAL SEGREGATION OF YOUNG ERYTHROCYTES

A POSSIBLE METHOD OF GENOTYPING THE TRANSFUSED PATIENT

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On certain occasions the need arises to discover the blood group genotype (more correctly phenotype) of a patient who has already been transfused. Some patients, for example, appear to form antibodies more actively than the general population, and in them it may be desirable to avoid transfusing all blood but that of the identical genotype. If they have already been transfused there is a mixture of cells in their circulation, and, even with a knowledge of donor genotypes, it may be impossible to uncover the patient's own contribution.

A more modern aspect of this problem is the attempt to treat hypoplastic anaemia or leukaemia with transfused haemopoietic cells. To give the maximum chance of success such cells should be of identical genotype except in so far as a single minor difference may be useful for the detection of foreign circulating cells, and is thereby an index of success.

In these circumstances, rather than wait three months or more until all previously transfused erythrocytes are eliminated, the following method may be tried. It depends, in essence, upon the centrifugal segregation of the younger and lighter circulating cells which, with certain exceptions later described, are derived from the subjects' own centres of haemopoiesis.

Evidence to show the possibilities and limitations of the method is presented, the data being of three kinds: (1) Serological analyses of known donor-recipient blood-group mixtures; (2) studies to show the concentration of reticulocytes by centrifugation; (3) studies on the gravitational behaviour of ^{51}Cr -tagged erythrocytes after transfusion.

Methods

For all studies in which ^{51}Cr -tagged cells were used 24 ml. of blood was collected into heparin. For serology and in some of the reticulocyte analyses

3.8% sodium citrate (0.5 ml. in 5 ml. blood) was the anticoagulant.

As soon as possible after collection 5–20 ml. of whole well-mixed blood was centrifuged for 20 minutes at 2,400 to 2,700 r.p.m. (c. 2,000 g). The plasma was removed and the upper quarter of the volume of packed red cells was carefully pipetted off. This constitutes the "first top layer." A pipette plunged to the bottom of the tube then removed the lower quarter of cells, the "bottom layer." An aliquot of the "first top layer" was resuspended in the plasma and spun as before but in a narrow tube. A "second top layer" is thus obtained, and from this, using a precipitin or haematocrit tube, a "third top layer" can be similarly derived. The quantity of this is enough for reticulocyte counts or agglutination tests but not for chromium counting.

Reticulocyte counts were performed on smears counterstained with May-Grünwald Giemsa. The number of cells counted ranged from 500 to 5,000 according to the abundance or scarcity of reticulocytes.

In the chromium experiments 20 ml. of blood in acid-citrate-dextrose was tagged with 60–100 μc . of ^{51}Cr . For valid comparison of the radioactivity of each sample the haemoglobin concentration was determined and the results (corrected for decay) are expressed first as counts/sec./g. Hb, and then this figure is converted to a percentage of the whole-blood activity of the first sample.

In the serological studies cells from whole blood and from each layer were washed in saline before being tested by standard agglutination techniques.

Absolute values were determined on fresh blood samples with sequestrene as anticoagulant.

Results

Serological Analyses of Known Donor-Recipient Blood Group Mixtures.—Eighteen patients were found who in the course of treatment received blood which had one or more detectable antigens lacked by the recipient. Following transfusion one to three samples of blood were taken at intervals and were tested with sera known to give negative results with the pure recipient blood.

TABLE I

ANALYSES OF BLOOD-GROUP MIXTURES AND EFFECT OF CENTRIFUGATION ON SUCH MIXTURES AT INTERVALS AFTER TRANSFUSION

Case No.	Recipient Group	Donor Group	Quantity (Units)	Storage	1	2	3
1	dd	D+	2	7 days	+++, ++, ++, +, 2d	+++, +++, ++, ++ (1.2% retics), 5d	
2	ēē, MM	E+(2) N+(2)	3	10-22 "	(E) +++, ++, W (N) ++, ++, W (42% retics) } 3d		
3	dd	D+	6	7 "	+++, W, neg., neg., 1d	+++, W, neg., neg., 7d	
4	ēē	E+	1	20 "	+, +, W, neg., 2d	+, W, W, neg. (10.2% retics), 10d	
5	ēē	C+	4	14-18 "	+++, ++, ++, 3d	+, +, +, ++ (8.9% retics), 7d	+, W, W, W (25% retics), 14d
6	dd	D+	2	5 "	+++, W, W, neg. (10.3% retics), 3d	+, W, neg., neg., 10d	
7	ēē	C+	1	?	+++, W, neg., neg., 3d	+++, W, W, neg., 9d	
8	dd	D+	1	15 "	+++, ++, W, ++, 4d	+, +, +, +, 10d	+, +, +, +, 17d
9	dd	D+	2	8 "	+++, +, W, 3d	+++, +, W, neg. (36.6% retics), 9d	
10	ēē	C+	1	13 "	+++, +, +, +, 2d	+++, +, +, W, 13d	+++, +, W, neg., 21d
11	NN	MN	1	23 "	+++, +, W, W, 6d	+++, +, W, neg., 13d	
12	ēē	E+	1	17 "	+, W, neg., neg., 6d	+, W, neg., neg., 14d	
13	MM	MN	1	?	+++, +, W, W, 3d	+++, W, neg., neg., 10d	+++, W, W, W, 17d
14	NN	MM	1	20 "	+++, ++, ++, ++, +, 3d	+++, +, +, +, ++, 9d	+++, ++, ++, ++, 16d
15	MM	MN	2	11-14 "	+++, +, W, W, 2d	+++, +, +, W, 10d	
16	dd	D+	3	20 "	+++, W, neg., neg., 1d	+++, W, neg., neg., 4d	
17	MM	MN	2	14 "	+++, +, W, neg., 10d	+++, ++, +, W, 17d	
18	dd	D+	2	14-20 "	+++, W, neg., neg. } 2d	+++, +, W, neg., neg., 14d (16d)	+++, +, neg., neg., 21 (23d)
			3 (2d later)	10-16 "	+++, W, neg., neg. } 7 (9d)		

In each column of analysis is shown (left to right) the reactions in whole blood, "first top layer," "second top layer," "third top layer" (in some), and the interval in days after transfusion. The reticulocyte percentage refers to the most refined top layer.

The results are listed in Table I. The interval after transfusion is indicated for each test, and beside this the degree of agglutination is shown for the whole blood and successive "top layers." The figure for reticulocytes in six cases refers to the percentage of these cells in the most refined top layer tested. Three points deserve attention: (1) The length of storage time of the transfused blood is not a factor influencing the rate or degree of segregation. (2) The amount of blood given does not always determine the degree of agglutination in the mixture. Thus in Case 10 one bottle of C+ blood gave rise to a +++ agglutination two days later, whereas in Case 5 4 pints given with the same donor-recipient combination and tested with the same serum gave no more than a ++ reaction at three days fading to a weak agglutination at 14 days. In partial explanation it should be stated that the degree of agglutination was measured by the size

of the agglutinates rather than by the proportion of free and agglutinated cells. The dosage effect of the C antigen may also be a relevant factor. (3) In 14 cases there was satisfactory separation of cells so that in the topmost layer the patient's true genotype was revealed. In four cases separation was inadequate although in two of these the "top layer" reaction for the foreign antigen was weaker than in the mixture. The lapse of time (two to five days in Case 1; three to 16 days in Case 14) did not appear to increase the chances of effective separation, and in Case 1 this may possibly be due to slow erythrocyte production during this period, the reticulocytes on the fifth day being 0.1% in whole blood, 1.2% in the "third top layer." In one of the other unresolvable cases (Case 8) time appeared to be a contrarily acting factor, initial separability giving way to a weakly reacting homogenous cell mixture. In Case 5 a uniform but weakening

TABLE II
CONCENTRATION OF RETICULOCYTES BY CENTRIFUGATION

Anti-coagulant	Reticulocyte Ratios						No. of Estimations	No. of Patients
	1st Top Layer Whole Blood		2nd Top Layer Whole Blood		3rd Top Layer Whole Blood			
	Range	Mean	Range	Mean	Range	Mean		
Citrate ..	1.0-3.5	2.4	2.8- 8.0	5.1	7.0-16.0	11.1	38	21
Heparin ..	1.8-4.2	2.6	2.3-13.0	5.5	—	—	43	11

distribution of antigen was found despite a reasonable concentration of reticulocytes in the upper layers.

Separation of Reticulocytes.—Progressive concentration of reticulocytes can be achieved by the method of centrifugation described above. In 81 blood samples from 32 patients in which two or more top layers were analysed only one failed to show a conspicuous increase in the reticulocyte content. This example and eight typical results are illustrated in Fig. 1. Table II shows that there is some variation in the degree of concentration, but with the technique used the increase from stage to stage is roughly logarithmic. The variation occurs even in different samples from the same patient, but it appears to be irrespective of the initial number of reticulocytes. There was no obvious distinction in behaviour between cells with different amounts of cresyl-blue staining material.

Studies with ^{51}Cr -labelled Erythrocytes.—Before describing the results it is necessary to explain the precise objective of these studies. An ordinary fresh blood sample labelled with ^{51}Cr will contain cells of all ages. After reinjection, as these cells age, any young cells in the blood will be unlabelled and there may

FIG. 1.—Concentration of reticulocytes by progressive centrifugation in nine typical cases and one exceptional case.

FIG. 2a, b.—Survival of ^{51}Cr -tagged autologous erythrocytes in two patients and the effect of centrifugation on the concentration of tagged cells. (Results expressed as a percentage of the initial whole blood activity in counts per second per g. Hb.) Whole blood, ●—●; "bottom layer", ○—○; "first top layer," X—X; "second top layer," +—+.

thus appear on centrifugation an upper layer of cells free from radioactivity.

The cells chosen for labelling could be either autologous or from a donor, but, since our aim was to learn if the young cells from within a mixed population could be distinguished, all but two of these studies were made with donor blood, fresh or stored, being in six cases part of a larger transfusion. This, as will be seen, raises complications. Multiple transfusion, variations in storage time, and the fact that all 11 patients were suffering from malignant disease, haemorrhage, or surgical trauma must be taken into account.

The results of the experiments with autologous labelled cells are presented in Fig. 2. Both were

FIG. 3a and b

FIG. 3d

FIG. 3e

FIG. 3a-i.—Survival of ^{51}Cr -tagged donor erythrocytes in nine transfused patients.

DONOR Hb: 15.1 g. MCHC: 33.5% (60 days after)
 RECIPIENT Hb: 17.3 g. MCHC: 33% (90 days after)

FIG. 3f

DONOR Hb: 12.4 g. MCHC: 32% (80 days after)
 RECIPIENT Hb: 12.2 g. MCHC: 33.5% (70 days after)

FIG. 3g

DONOR Hb: 8.9 g. MCHC: 28%
 RECIPIENT Hb: 13.1 g. MCHC: 32%

FIG. 3h

DONOR Hb: 9.7 g. MCHC: 29.5%
 RECIPIENT Hb: 14.9 g. MCHC: 34%

FIG. 3i

cases of fairly advanced malignant disease. They were selected because, while similar in most respects, one (Fig. 2a) had consistently about 10 times as many reticulocytes as the other (Fig. 2b), a difference which is only explained in part by a shorter mean cell life, i.e., 50% elimination at 20 days compared with 28 days.

It is apparent that the higher reticulocyte concentration is associated with a more rapid clearing of labelled cells from the top layers, but changes must also be occurring in the immediate post-reticulocyte phase. Thus in Fig. 2a the ratio top layer activity/whole blood activity is constantly decreasing at least up to 15 days while a similar but lesser change is seen in Fig. 2b. Clearly the reticulocytes alone cannot be responsible for this alteration, and it suggests that the gain in density of the cells proceeds at an ever-decreasing rate which after about 15 days is very slow.

In the studies where donor cells were labelled (Fig. 3a-i) there can be distinguished three main groups of cases. In the first three cases (Fig. 3a-c) there is complete segregation of donor cells to the lower layers from the first (24-hr.) sample onwards, while at the other extreme are four cases (Fig. 3f-i) with no satisfactory clearing of the upper layers during the course of the experiments. In between come two cases, in one of which (Fig. 3d) the labelled cells are most concentrated in the middle layers while in the other (Fig. 3e) there is a rapid shift within two weeks from the upper to the lower layers.

Obviously some other hypothesis is needed to account for such very different results, and this may be partially provided by a consideration of the relative haemoglobin concentrations in the donor and recipient corpuscles. The values were determined for 17 out of 18 bloods, but in seven cases after the experiments. Clearly the latter data are of limited value, but, as there is no reason to suspect any great change since the time of the experiments, they are of some use if they confirm, as they do, the better-established data.

The evidence, so far as it goes, shows that the initial segregation is in accordance with the relative M.C.H.C. of donor and recipient bloods. On the first day, that is, the cells with the higher M.C.H.C., and hence presumably heavier, whether of donor or recipient origin, are more concentrated in the bottom layers. Thereafter, in some cases (especially, for example, Fig. 3e), an adjustment of some sort may occur to change this relationship in addition to the ageing effect previously noted.

Discussion

It might be thought a tautology to say that the centrifugal behaviour of a cell depends upon its density, but, in fact, this is not always true. If sedimentation is slow, as under the influence of gravity only, there is time for aggregation of a proportion of cells to occur, and this event, which depends upon surface characteristics, will cause the more rapid precipitation of aggregated cells. Simple sedimentation was, indeed, found by Stephens (1939) to be a useful way of separating young, less readily aggregated erythrocytes, but in the different circumstances of our experiments this method has proved ineffective.

Most workers (for references see Marks and Johnson, 1958), relying on density differences, have used centrifugation with or without the addition of albumin to the suspending medium. The factors which render this separation effective are:

- (1) The greater water content of the young cell which is reflected in its greater size with consequent dispersal of protein and other material.

- (2) The absolute increase of haemoglobin in the mature cell, which one must assume, since haemoglobinization is still occurring in the reticulocyte (Walsh, Thomas, Chow, Fluharty, and Finch, 1949).

- (3) A decrease in lipid content of the maturer cells (Pranker, 1958).

Borun, Figueroa, and Perry (1957) using ^{59}Fe have produced some interesting data indicating that density is, in fact, a function of erythrocyte age but that considerable individual variation occurs in the degree of this relationship and also in its timing. Nevertheless it is clear that the greatest increase in density occurs early in the life of the cell.

Bearing in mind these sources of variation, it is scarcely surprising that one cannot, when dealing with cell mixtures, always rely on centrifugation to bring the young erythrocytes to the top. But our studies show that the most adverse conditions for separation are those where cells with a low haemoglobin concentration are transfused to a recipient of more fully haemoglobinized cells. Luckily this is the opposite of what usually occurs in clinical practice (our experiments with chromium-labelled cells are heavily biased by selection), and, as shown in the serological studies, in the majority of cases the recipient genotype is revealed in the top layer.

The method has been successfully used in one patient of unknown genotype, and the test proved

correct after a lapse of time when all transfused cells had been eliminated; also in one case to detect erythrocyte chimerism as a result of transfusion of haemopoietic cells. By testing the young cells as they emerge into the circulation the development and cessation of function of donor marrow cells can be followed with much greater precision than could ever be obtained by the use of whole blood alone.

Alternative procedures should be mentioned. Cathie (1946) has described the genotyping of bone marrow cells, and another approach to the problem is suggested by the work of Marks and Johnson (1958) whereby the osmotic resistance of young erythrocytes could be exploited. Both methods would seem to have their own difficulties. Meanwhile those who find occasion to employ centrifugal separation should take note of the pitfalls.

Summary

Observations have been made on the separation of erythrocytes by centrifugation. Young cells being lighter tend to spin to the top, but in mixtures caused by transfusion other factors, especially the corpuscular haemoglobin concentration, may sometimes obscure this tendency. Nevertheless centrifugation can be recommended at least as an adjunct in the problem of genotyping cell mixtures.

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